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Effects of anthocyanidins on myogenic differentiation and antioxidant defense in primary myogenic cells isolated from rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

There is increasing interest in using plant-derived extracts to promote growth and health in finfish species in recent years. Elucidating the effects of plant secondary metabolites on skeletal muscle growth signaling will contribute to an improved understanding of the effects of feeding carnivorous fish diets supplemented with plant extracts on fish somatic growth. Dietary intake of anthocyanins, a type of flavonoid widely distributed in plants, has long been associated with beneficial effects in both human and animal health; however, their effects in finfish are largely unknown. We conducted an experiment to test the effect of three doses (treatments A, B and C; 1×, 2.5× and 10×, respectively) of a mixture of three types of anthocyanidins (peonidin, cyanidin and pelargonidin chloride) on the expression of several genes in primary myogenic cells isolated from the skeletal muscle of rainbow trout (*Oncorhynchus mykiss*) after 24 h of treatment. The genes of interest analyzed are involved in myogenic programing (*pax7*, *myoD* and *myogenin*), Notch signaling (*her6* and hey2) and antioxidant enzymes (*sod1*, *cat* and *gpx1*). Significantly greater expression of *pax7* in cells under treatment B compared with the untreated cells was detected. Although no differences in expression of myogenic regulatory factors, *myoD* and *myogenin* between test groups or the control were detected, a trend toward significantly lower expression in all groups tested compared with the control group was observed. Moreover, significantly higher expression levels of *her6* and *hey2* in cells under treatments A and B compared with untreated cells were detected. Although no significant differences in the expression of *cat* and *sod1*, significantly greater expression in *gpx1* in all treated groups compared with the control group was detected. Collectively, we demonstrated that anthocyanidins enhance the expression of *gpx1* in primary myogenic cells, thereby contributing to skeletal muscle tissue defense against oxidative stress in finfish species. Further, anthocyanidins appear to delay myogenic differentiation in primary myogenic cells by up-regulating the expression of *pax7* while decreasing myogenic regulatory factors in a Notch signaling-dependent interaction. Whether this effect results a reduced growth performance and/or an increase in feed conversion ratio in fish fed diets supplemented with plant extracts rich in anthocyanins or anthocyanidins needs further study, and the need to better define the potential effects of different polyphenol classes in myogenic differentiation on primary myogenic cells from carnivorous fish is warranted.

Statement of relevance: The study contributes to increase our understanding regarding the effect of plant-derived secondary metabolites such as anthocyanidins on myogenic program and antioxidant enzyme defense in differentiating myogenic cells from carnivorous fish. We have demonstrated that anthocyanidins may delay the progress of the myogenic differentiation process and promote antioxidant defense expression in myogenic cells.

1. Introduction

During the last several decades, research in nutrition of carnivorous teleost species has been predominantly focused on the effects of feeding fish either total or partial plant ingredient-based diets on growth, health and product quality in fish (Burel et al., 2000; Gomes et al., 1995;

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Kaushik et al., 1995, 2004; Overturf and Gaylord, 2009; Snyder et al., 2012; Wacyk et al., 2012). This is especially true due to the steady increase in prices of marine-derived aquafeed ingredients, fishmeal and fish oil Naylor et al., (2009). As inclusion levels of plant ingredients in carnivorous fish diets increase, it is necessary to understand the effects of feeding diets containing phytocompounds in these species. In this regard, most of the research in this field has been to alleviate the detrimental effects of phytocompounds acting as anti-nutritional factors in finfish species, mainly carnivorous species (Krogdahl et al., 2010). Nevertheless, recent evidence has demonstrated that phytochemicals including flavonoids, alkaloids, terpenoids, tannins, glycosides, steroids and essential oils, elicit a plethora of beneficial effects in finfish species (Bennetau-Pelissero et al., 2001; Chakraborty et al., 2014; Leiro et al., 2004; Perez-Escalante et al., 2012; Reverter et al., 2014; Saito et al., 2002). Therefore, there is growing interest in the potential use of plant-derived extracts for disease control as an alternative to chemical treatments as well as their use as promoters of appetite and growth performance in finfish species (Reverter et al., 2014).

Anthocyanins, a flavonoid-polyphenol subclass, are found in several vegetables such as purple potatoes, purple carrots, purple corn, black soybean and purple beans (Ha et al., 2010; Hwang et al., 2011; Poudyal et al., 2010; Ramos-Escudero et al., 2012; Zhang et al., 2013a). Previous studies have reported potential health benefits, such as antioxidant, cardio-protective, anti-inflammatory and anti-carcinogenic effects, from dietary intake of anthocyanins in humans and other mammals (Galvano et al., 2004; Vennat et al., 1994; Wallace and Giusti, 2013; Whitehead et al., 1995). We recently demonstrated beneficial effects such as higher plasma antioxidant potential and greater gene expression of glutathione peroxidase 1 (*gpx1*) in erythrocytes of trout fed a diet supplemented with purple corn extract, a natural source of anthocyanins (Villasante et al., 2015). Additionally, in a previous study, Perez-Escalante et al. (2012) observed significant improvement in biometric parameters such as higher specific growth rate and lower feed conversion ratio as well as higher survival in goldfish (*Carassius auratus*) fed a diet supplemented with roselle anthocyanin extract in comparison to a control group. Whether this growth promotion observed in fish fed the anthocyanin extract was due to a stimulatory effect on myogenesis needs to be further explored in fish species of aquaculture importance. In this regard, previous studies have reported that polyphenols including resveratrol and (−)-epicatechin promote myogenic differentiation in mammalian-derived C2C12 myoblasts by up-regulating the expression of several myogenic regulatory factors including *myf5*, *myoD*, *myogenin* and *mef2* (Gutierrez-Salmean et al., 2014; Kaminski et al., 2012; Laçon; et al., 2012; Montesano et al., 2013). In agreement with this statement Myburgh et al. (2012) observed an accelerated skeletal muscle recovery after in vivo administration of grape-derived proanthocyanidolic oligomers in rats with contusion-induced damage. The authors observed that an accelerated activation and proliferation of satellite cells as well as the earlier expression of the fetal isoform of myosin heavy chain (MHCf) contributed to the faster recovery effect observed in rats fed the polyphenol supplemented diet compared to the control. However, recent evidence suggests that pro-differentiation effect of polyphenols such as resveratrol depends on the dose and the reductive–oxidative balance status of the myogenic cell. A low resveratrol dose promoted in vitro muscle regeneration and attenuated the impact of reactive oxygen species (ROS), while high doses reduced plasticity and metabolism induced by oxidative stress in C2C12 myoblasts (Bosutti and Degens, 2015). The mechanism involved appears to be intricate and complex, involving the role of both free radicals acting as signaling molecules and miRNAs including miR-133, miR-20b and miR-149 regulating the expression of pro-myogenic genes (Kaminski et al., 2012; Laçon et al., 2012; Gutierrez-Salmean et al., 2014; Montesano et al., 2013). However, the potential modulatory effect of polyphenols including flavonoids such as anthocyanins or their aglycon forms (anhtocyanidins) in myogenic differentiation in fish species of aquaculture interest has not yet been addressed.

The paired box protein 7 (Pax7) is a member of the paired box transcription factor family, which plays a crucial role during proliferation of and maintenance of an effective satellite cell pool (myogenic progenitor cells) essential for growth, repair, and maintenance of skeletal muscle in juvenile and adult mammals (Bentzinger et al., 2012; von Maltzahn et al., 2013; Zammit et al., 2006). Myogenic regulatory factors (MRF) including *myoD* and *myogenin* exhibit different expression patterns during myogenesis. *MyoD* is up-regulated during recruitment and determination of satellite cells as well as proliferation of myoblasts while *myogenin* is expressed during myoblast terminal differentiation into myocytes, regulating the expression of myotube specific genes (Olguín and Pisconti, 2012; Pownall et al., 2002). Determination of *pax7*/*myoD* ratio is an important indicator of satellite cell fate, identifying progression toward differentiation into myoblasts or promotion of satellite cell self-renewal (Olguin et al., 2007; Chapalamadugu et al., 2009; Olguín and Pisconti, 2012).

The Notch signaling pathway plays a crucial role during development (Artavanis-Tsakonas et al., 1999) but its biological importance goes well beyond that. Notch signaling activation is shown to be crucial in avoiding certain muscular dystrophic phenotypes and promotes muscle regeneration in mice (Lin et al., 2013). Bjornson et al. (2012) demonstrated that Notch signaling promotes both self-renewal of skeletal muscle satellite cells and maintenance of normal adult myogenesis in mice. Constitutive activation of Notch signaling is known to induce self-renewal of skeletal muscle satellite cells via up-regulation of *pax7* in C2C12 myoblasts (Wen et al., 2012). Considering the abovementioned, we analyzed the effect of three types of anthocyanidins (peonidin, cyanidin and pelargonidin chloride) which are the aglycons (non-glycoside form) of anthocyanins, on the expression of genes involved in cell antioxidant defense, namely catalase (*cat*), superoxide dismutase 1 (*sod1*), glutathione peroxidase 1 (*gpx1*) and the nuclear factor (erythroid-derived 2)-like 2 (*nrf2*) and genes associated with myogenic differentiation including *pax7*, *myoD* and *myogenin* and two target genes of the Notch signaling pathway, namely Hairy/enhancer-of-split related with YRPW motif protein (*hey2*) and Hairy/enhancer-of-split related 6 (*her6*), an ortholog of mammalian *hes1* (Davis and Turner, 2001; Liu et al., 2006). The findings of this study provide novel insight with regard to the potential modulatory role of anthocyanidins in myogenic program in primary myogenic cells isolated from carnivorous fish. Although polyphenols including anthocyanins and anthocyanidins are found in several vegetables and fruits that are not common ingredients for aquafeeds, the use of extracts derived from low-cost agroindustry by-products rich in these compounds could offer a cost-effective option to include functional ingredients in aquafeeds that could contribute to improve growth, health and final product quality in finfish species under intensive culture.

2. Material and methods

2.1. Anthocyanidin mixture preparation

An anthocyanidin mixture of three types of commercial anthocyanidins aglycons, peonidin chloride (A385015M005, Fisher Scientific, Houston, TX, USA), cyanidin chloride (79457, Sigma-Aldrich, St. Louis, MO) and pelargonidin chloride (P1659, Sigma-Aldrich, St. Louis, MO) was prepared using nanopure water as the solvent. The final stock solution concentrations of peonidin chloride, cyanidin chloride and pelargonidin chloride were 50 mM, 20 mM and 15 mM respectively. The anthocyanidin proportions were similar to that measured in a sample of purple corn extract analyzed previously in our lab (Villasante et al., 2015).

2.2. Cell culture

2.2.1. Myogenic cell isolation

All experimental procedures were conducted following the guidelines of the Institutional Animal Care and Use Committee at the